

Purification, characterization and evaluation of insecticidal activity of trypsin inhibitor from *Albizia lebbeck* seeds

Pratima Sharma • Amarjit K Nath • Reena Kumari • SV Bhardwaj

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Abstract: A Bowman-Birk inhibitor with activity against gut proteases of *Helicoverpa armigera* was extracted in 0.1 M sodium phosphate buffer from defatted seed flour of *Albizia lebbeck*. It was purified to 29.62 folds with 51.43% recovery using ammonium sulfate precipitation, gel filtration chromatography on Sephadex G-100 column and ion exchange chromatography on DEAE-Sephadex A₅₀. The purified protein had a molecular weight of 12,303 daltons as determined by SDS-PAGE. It was found to be heat stable up to 60°C and had two pH optima of 7.5 and 9.0. The inhibitor exhibited non-competitive pattern of inhibition with a low K_i value of 0.2 μ M. The inhibitor was found to be susceptible to varying concentrations of reducing agents like DTT and 2-mercaptoethanol, thereby indicating the role of disulphide bridges in maintaining its three dimensional structure and stability. The purified inhibitor caused mortality and suppressed larval growth of *Pieris brassicae* larvae. It was also found to be effective against gut trypsin extracted from *Spodoptera littoralis*. The sequence of the genes encoding for such inhibitors can be determined and the genes expressing protease inhibitors can be used in vegetable crops to confer resistance against insect pests and other plant pathogens.

Keywords: *Albizia lebbeck*; trypsin inhibitor; insecticidal activity purification; *Pieris brassicae*

Introduction

The use of transgenic crops has been rapidly advancing during the past decade with the discovery of effective plant genes that can be transferred and offer resistance to crop plants against pests and pathogens. Proteinase inhibitors (PIs) are potential

candidates for biocontrol of insect pests since insect digestive proteinases are promising targets towards control of various insects. PIs have been found to be effective against many Coleopteran (Elden 2000) and Lepidopteran (Liao et al. 2007) insect pests. Therefore, a thorough understanding of insect gut proteinases is required for the development of pest resistant transgenics using PIs. Several plant species have been transformed with the protease inhibitor genes for controlling insect pests. PIs have also been used to engineer resistance against viruses in transgenic plants (Ussuf et al. 2001). These have the potential to counteract many of the inherited disorders such as emphysema and epilepsy. They can also interfere with the life cycle of many viruses and may help prevent many viral disorders. Synthetic PIs currently form a part of the combinational therapy against AIDS and have potential to be used as drugs against many other diseases.

PIs are, in general, small regulatory proteins showing inhibitory activity against all the four mechanistic classes of proteolytic enzymes i.e. serine, cysteine, aspartic and metallo proteinases. Occurring commonly and copiously in the leguminous seeds, PIs have been classified into two distinct families of Kunitz and Bowman Birk type of inhibitors on the basis of molecular mass and disulfide bonds pattern. Kunitz type inhibitors have a single polypeptide chain with a molecular mass ~20,000 daltons, two intra chain disulfide bridges and lower cysteine content. Bowman-Birk inhibitors are also single chain polypeptides but smaller in size. Their molecular mass ranges from 7,000 daltons to 10,000 daltons and possess seven disulfide bridges and two active domains for trypsin and/or chymotrypsin. They are characterized by high cysteine content (Richardson 1991). PIs act as antimetabolic proteins, which interfere with the digestive process of insects. They inhibit the activity of the gut proteinases of insects and reduce the quantity of proteins that can be digested and also cause hyper production of the digestive enzymes, which enhances the loss of sulfur amino acids, as a result of which the insects become weak, with stunted growth and ultimately die. The mechanism of action minimizes the possibility of developing resistance in insects and reduces crop damage (Shulke and Murdock 1983). Among the studied PIs, trypsin inhibitor is perhaps the most important, since trypsin not only activates several of the

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Pratima Sharma • Amarjit K Nath (✉) • Reena Kumari • SV Bhardwaj
Department of Biotechnology, UHF, Nauni, Solan, India.
Email: amarjitnath@yahoo.com; Phone no.: 09816012296, 01792-252639

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digestive proteases which are secreted as proenzymes in the digestive tract, but also regulates their secretion. Trypsin inhibitors present in different plants show variable levels of activity towards the proteolytic enzymes of target insects (Broadway and Duffey 1986; Purcell et al. 1992).

Although innumerable plant PIs have been documented till date, many of which are part of successful transgenes either against insect pests or as a therapeutic drug molecule, much exploration still remains to be undertaken since each of such molecules may differ not only in their potency but also in their mode of action. The present study thus aims at purifying and characterizing a trypsin inhibitor from the seeds of *Albizzia lebbeck*, a Mimosoid leguminous tree.

Materials and methods

Seeds of *Albizzia lebbeck*

Seeds of *A. lebbeck* were collected from Bilaspur region of Himachal Pradesh, India.

Chemicals

The chemicals procured from Sigma Aldrich (USA) included Bovine pancreas trypsin, Sephadex G-100, DEAE-Sephadex (A_{50}) and BApNA. Molecular weight markers (SDS-markers 10 kDa – 225 kDa) were purchased from Merck (Germany). All other chemicals and reagents used were of analytical grade.

Insect larvae

The lab cultured larvae of *Helicoverpa armigera* for isolation of gut protease were obtained from the Department of Entomology and Apiculture, Dr. Y. S. Parmar University of Horticulture and Forestry. Larvae of *Spodoptera littoralis* were obtained from the fields of Department of Floriculture and Landscaping, Dr. Y. S. Parmar University of Horticulture and Forestry. The leaves of cabbage with eggs of *Pieris brassicae* were collected from cabbage fields of Kotkhai region of Shimla, Himachal Pradesh, India.

Preparation of crude extract

Protein was extracted following the methods of Hajela et al. (1999) and Maggo et al. (1999). The seeds of *A. lebbeck* were ground to make fine powder. The flour obtained was defatted with acetone (1:10 w/v) and air dried. Defatted flour (100 mg) was extracted for 30 min in 10 ml of extraction buffer (0.1 M sodium phosphate buffer, pH 7.5) at room temperature by constant stirring on a magnetic stirrer. The suspension obtained was centrifuged at 1000 \times g for 30 min at 4°C. The supernatant was used for determination of trypsin inhibitor activity against bovine pancreas trypsin.

Purification of trypsin inhibitor

The crude extract of *A. lebbeck* seeds was used for inhibitor purification. The supernatant was fractioned by ammonium sulfate precipitation (20%–80%). The protein was allowed to precipitate overnight at 4°C. The precipitated protein was collected by centrifugation at 10,000 \times g for 20 min at 4°C and the pellet was resuspended in minimum volume of 0.1 M sodium phosphate buffer (pH 7.5) and dialyzed. The dialyzed (20%–80%) ammonium sulfate fraction was loaded on the Sephadex G-100 (31 \times 2.5 cm) and eluted with 3 bed volumes of 0.1 M sodium phosphate buffer (pH 7.5).

The fractions with trypsin inhibitory activity were pooled and then subjected to ion exchange chromatography on DEAE-Sephadex (A_{50}). The column was first eluted with distilled water to wash out the unbound proteins. The bound proteins were eluted with linear salt gradients of 0.1–0.4 M KCl. The active fractions eluted with 0.2 M KCl were pooled and concentrated against solid sucrose at 4°C and used for further studies.

Trypsin inhibitory assay

The activity of trypsin inhibitor was assayed according to the method described by Hajela et al. (1999) with slight modification. One ml of trypsin solution was mixed with 0.1 ml of diluted trypsin inhibitor and incubated in a metabolic shaking water bath at 37°C for 10 min, followed by the addition of suitable volume of buffer-I (prepared fresh by mixing 10 ml of 0.1 M sodium phosphate buffer (pH 7.5), 4 ml of 0.1 M $CaCl_2$ and 6 ml of Distilled water) and 0.3 ml of BApNA (4 mg of BApNA dissolved in 0.1 ml of dimethyl sulphoxide (DMSO) and the final volume made to 1 ml by adding 0.9 ml of buffer-I). The reaction was stopped after 10 min by adding 0.5 ml of 30% acetic acid.

Blank was prepared by adding 0.3 ml of buffer-I instead of substrate BApNA whereas a positive control was maintained without the trypsin inhibitor and the volume of the reaction mixture was adjusted with buffer-I. To measure the trypsin inhibitor activity (TIA), optical density of test and control reaction mixtures was measured at 410 nm. Trypsin units inhibited (TUI) were calculated by comparing the absorbance measured for test reaction mixture and control reaction mixture. Decline of 0.01 optical density (OD) units per minute in case of test reaction as compared to the control reaction was taken as one trypsin unit inhibited (TUI).

Extraction and assay of larval midgut protease of *Helicoverpa armigera*

Midguts from actively growing, lab cultured fourth instar larvae were dissected out on ice and homogenized in 0.1 M phosphate buffer (pH 7.5) in prechilled test tube using a glass rod. The homogenate obtained was filtered through filter paper and the filtrate was then used for gut enzyme inhibition assay which followed the same procedure as described in the assay of trypsin inhibitor activity using larval midgut trypsin instead of bovine trypsin.

Electrophoretic analysis

The homogeneity of the purified protein was checked by native-PAGE (7.5%) as per the protocol of Davis (1964) and SDS-PAGE (12.5%).

Characterization of purified inhibitor

In all the characterization experiments the trypsin inhibition assay was carried out as described earlier.

Molecular weight determination

The molecular weight of purified inhibitor was estimated by SDS-PAGE using standard SDS molecular weight markers (10 kDa – 225 kDa).

Effect of inhibitor concentration

The effect of inhibitor concentration on trypsin activity was studied by varying the concentration of inhibitor in the inhibition assay. The inhibitor concentration varied from 0.67 μ g to 6.66 μ g in the inhibition assay.

Effect of temperature on stability of trypsin inhibitor

The effect of temperature on stability of trypsin inhibitor was studied by incubating the inhibitor for 10 min at 30, 40, 50, 60, 70, 80, 90°C and measuring the inhibitor activity.

Effect of pH

Different buffers viz., acetate buffer (pH 4.5 to 5.5), phosphate buffer (pH 6.0 to 8.0) and Tris buffer (pH 8.0 to 9.5) were used in the reaction mixture. Appropriate controls for respective buffers were taken and trypsin inhibitor activity was then monitored.

Determination of K_M value and nature of inhibition

To determine the nature of inhibition, two different fixed inhibitor concentrations (2.5 μ g and 5.75 μ g) were used. Lineweaver Burk (1934) plot was plotted using different concentrations of substrate in the presence and absence of inhibitor and K_M value was determined.

Determination of K_i value

Dixon's plot was plotted using different concentrations of inhibitor at two fixed concentrations of substrate (1.84 mM and 4.6 mM). The K_i value was determined from the plot as described by Dixon (1953).

Effect of 2-Mercaptoethanol and Dithiothreitol (DTT)

Trypsin inhibitor was incubated with different concentrations of

2-mercaptopethanol and dithiothreitol for 1 h. Different concentrations of 2-mercaptopethanol used were 100 mM, 200 mM and 400 mM and that of dithiothreitol were 15 mM and 20 mM. The trypsin inhibition assay was then carried out using the above extracts.

Effect of trypsin inhibitor on larvae of *P. brassicae*: Feeding bioassay

Cabbage leaf discs were coated with 67 μ g of purified trypsin inhibitor. Feeding assay was conducted by feeding 10 larvae of *P. brassicae* on treated leaf discs, immediately after hatching and same number of larvae on cabbage leaf disc coated with distilled water was taken as control. The experiment was conducted in three sets. All the larvae were subsequently given fresh leaves after 24 h for three days. The per cent leaf area eaten and weight of faecal matter was recorded after 24 h.

Similarly, feeding bioassays were conducted using five days old larvae. The experiment was carried out in three sets. Four larvae were exposed per replication and 134 μ g of purified trypsin inhibitor was coated on leaf discs (6 cm diameter). The larvae were given fresh leaves after 24 h for two days. In control, the same numbers of larvae were placed on leaf discs coated with distilled water. The per cent leaf area eaten and weight of faecal matter were recorded after 24 h.

Effect of ALTI on gut protease of *Spodoptera littoralis*

The midguts of *S. littoralis* were surgically removed from the larvae and placed into a prechilled test tube having 0.1M phosphate buffer. Guts were homogenized and centrifuged at 10,000 \times g at 4°C for 30 min. The supernatant was used as source of trypsin enzyme in trypsin inhibitor assay.

Statistical analysis

All the laboratory experiments were carried out in three replications with duplicate for each replication. The CRD design was applied to the data of inhibitor extraction and t-test was applied to feeding bioassay.

Results

Trypsin inhibitor activity in seed extract of *A. lebbeck* was measured using BApNA (α -Benzoyl-DL-arginine-p-nitroanilide) as substrate and bovine pancreas trypsin as source of enzyme. The trypsin units inhibited (TUI) per mg seed flour weight were calculated to be 18.08 ± 0.04 . Also, initially the gut enzyme of *H. armigera* larvae was used as source of trypsin enzyme and the crude extract was tested for its trypsin inhibitor activity using BApNA as substrate. Trypsin units inhibited per mg seed flour weight were found to be 15.56 ± 0.184 . ALTI was purified to near homogeneity with 29.62 fold purification and 51.43% recovery (Table 1) by ammonium sulfate precipitation (20%–80%), gel filtration chromatography on Sephadex G-100 (Fig. 1) and ion exchange chromatography on DEAE-Sephadex A₅₀ (Fig. 2). In

ion exchange chromatography, the activity of ALTI was recovered in bound fractions and was eluted using a linear gradient of 0.2 M KCl. The purified inhibitor showed a single band in Na-

tive-PAGE and SDS-PAGE indicating it to be homogenously composed of a single polypeptide chain (Fig. 3).

Table 1. Purification of trypsin inhibitor from *Albizia lebbeck*

Step	Total TUI	Total soluble protein (mg)	Specific activity (TUI/mg protein)	Fold purification	Per cent yield (%)
Crude extract	18085.7	276.4	65.43	1.00	100
Ammonium sulfate precipitation	16785.7	121.2	138.49	2.12	92.81
Gel filtration chromatography	13378.8	66.6	200.89	3.07	73.97
Ion exchange chromatography	9302.4	4.8	1938	29.62	51.43

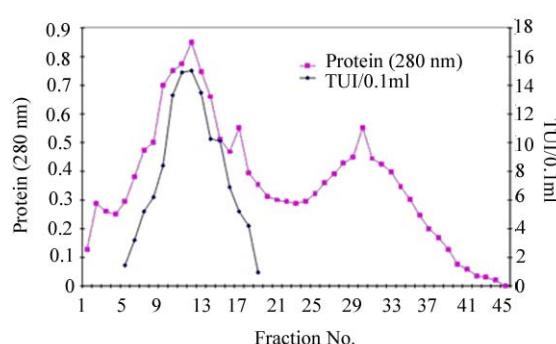


Fig. 1 Elution profile of trypsin inhibitor on Sephadex G-100 column

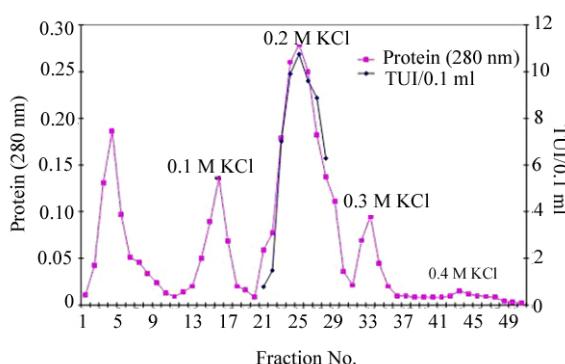


Fig. 2 Elution profile of trypsin inhibitor on DEAE-Sephadex (A₅₀)

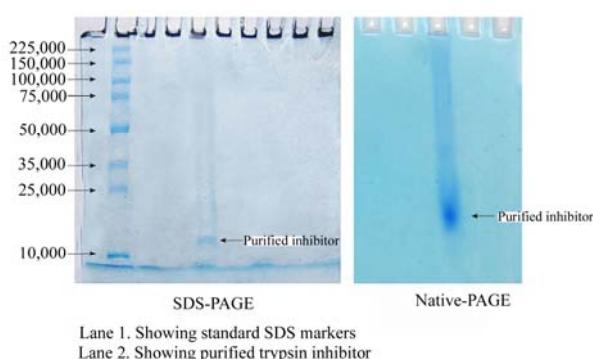


Fig. 3 Electrophoresis

Determination of molecular weight

The molecular weight of purified ALTI was 12,303 as estimated by SDS-PAGE using standard SDS molecular weight markers.

Kinetics of inhibition

The dissociation constant (K_i) value of ALTI was determined from Dixon's plot (Fig. 4) using BApNA as a substrate. The dissociation constant (K_i) value was found to be 0.2 μ M, which indicated high affinity of the inhibitor. The K_M value of 1.25 mM was obtained from Lineweaver-Burk plot (Fig. 5) and the inhibition was found to be of non-competitive type.

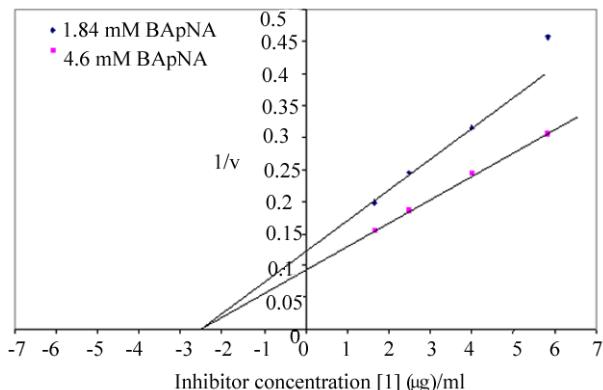


Fig. 4 Dixon's plot of trypsin activity in presence of different concentrations of inhibitor and two fixed concentrations of substrate

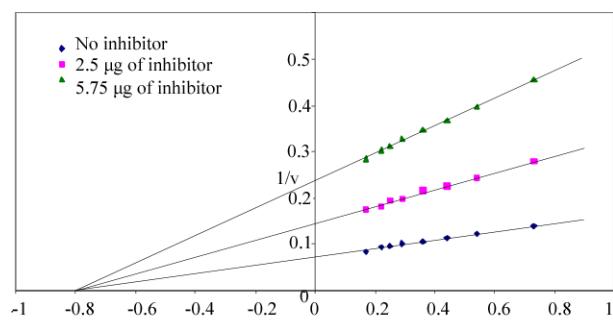


Fig. 5 Lineweaver-Burk plot of trypsin activity in presence of two fixed concentrations of inhibitor

Stability studies

In thermostability studies, the trypsin inhibitory activity was determined at temperatures ranging from 20–90°C. The purified inhibitor was found to be heat stable over a wide range of temperature (20–60°C). Above 60°C there was a slight decrease in inhibitor activity and 81.5% of the activity was retained at 70°C. Almost no inhibitory activity was observed at and above 80°C. While studying the pH stability, two pH optima of 7.5 and 9.0 were obtained. The inhibitory activity was also examined in the presence of reducing agents DTT and 2- mercaptoethanol. AIWI was found to be susceptible to varying concentrations of reducing agents. 29% trypsin inhibitor activity was left after one hour on incubation with 20 mM DTT and 5% trypsin inhibitor activity was left after one hour on incubation with 0.1 M 2- mercaptoethanol.

Feeding bioassay with larval *P. brassicae*

The feeding bioassay with newly hatched *P. brassicae* larvae showed that the larvae were sensitive to purified AIWI. The larvae that were fed on cabbage leaf discs of uniform area coated with 67 µg of purified inhibitor (Table 2, 3) showed a significant reduction in development and survival compared to larvae fed on control leaves. Reduction in larval development was judged solely from a reduction in size and excreta (Fig. 6). The percent leaf area eaten and faecal matter of the larvae fed on AIWI were significantly less than the control. Suppressed larval growth followed by 100 percent mortality was observed after two days when the larvae were fed on leaves coated with 134 µg of purified AIWI (Table 4, 5).

Table 2. Percent of leaf area eaten by larvae of *Pieris brassicae* (after 1st day of hatching)

Concentration of trypsin inhibitor (µg)	Per cent leaf area eaten		
	1st day	2nd day	3rd day
Control (0)	50.55 ± 1.21	66.553 ± 2.32	76.15 ± 1.26
Treatment (67)	8.973 ± 0.969	8.42 ± 1.00	5.95 ± 0.823
t value	26.811	22.947	46.63

Table 3. Faecal matter (mg) of larvae of *Pieris brassicae* (after 1st day of hatching)

Concentration of trypsin inhibitor (µg)	Faecal matter (mg)		
	1st day	2nd day	3rd day
Control (0)	1.6 ± 0.294	3.526 ± 0.20	4.63 ± 0.25
Treatment (67)	0.24 ± 0.034	1.303 ± 0.22	0.943 ± 0.045
t value	4.584	7.424	14.422

Table 4. Percent of leaf area eaten by larvae of *Pieris brassicae* (Five days old)

Concentration of Trypsin inhibitor (µg)	Per cent leaf area eaten	
	1st day	2nd day
Control (0)	67.756 ± 3.58	66.9733 ± 2.756
Treatment (134)	5.044 ± 2.53	3.2533 ± 1.132
t value	14.2897	21.380

Table 5. Faecal matter (mg) of larvae of *Pieris brassicae* (Five days old)

Concentration of Trypsin inhibitor (µg)	Faecal matter (mg)	
	1st day	2nd day
Control (0)	4.94 ± 0.39	6.32 ± 0.34
Treatment (134)	1.196 ± 0.1	0.73 ± 0.082
t value	9.257	16.054

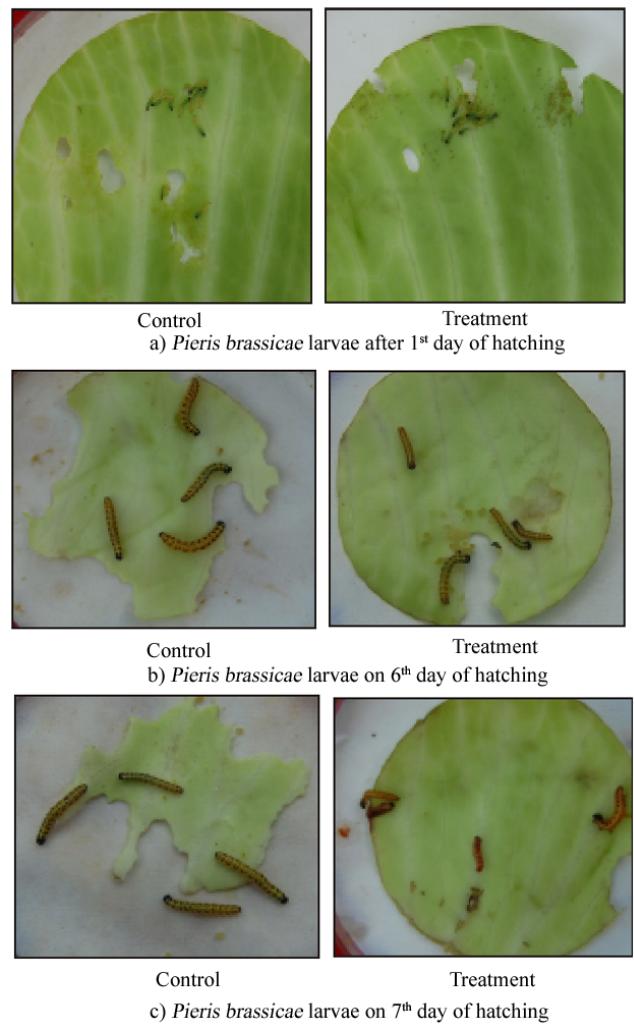


Fig. 6 Effect of trypsin inhibitor on larvae of *Pieris brassicae*

Effect of AIWI on gut protease of *Spodoptera littoralis*

Purified AIWI was tested for its trypsin inhibitory activity using BAPNA as substrate on the trypsin extracted from the gut of *Spodoptera littoralis*. Trypsin units inhibited per ml of purified inhibitor was found to be 52.6±0.24.

Discussion

Interest in protease inhibitors has increased in the last decade due to its potential biotechnological applications. In the present study,

a Bowman-Birk type trypsin inhibitor from *A. lebbeck* seeds was purified and characterized. In the present study, trypsin inhibitor present in crude extract of *A. lebbeck* seeds caused effective inhibition of gut proteases from the fourth instar larvae of *H. armigera*. Purified trypsin inhibitors from *A. kalkora* (Zhou et al. 2008) and mungbean (Kansal et al. 2008) were also found to be inhibitory towards gut proteases of *H. armigera*. Highly purified ATI was obtained with over 50% recovery following salting out with ammonium sulfate and several chromatographic procedures. Till date trypsin inhibitors have been purified and characterized from a variety of plant sources (Macedo et al. 2000; Oliveira et al. 2007; Kansal et al. 2008; Rai et al. 2008). The purified inhibitor protein was found to consist of a single polypeptide chain of 12,303 daltons as revealed by Native-PAGE and SDS-PAGE, which is consistent with the molecular masses of other trypsin inhibitors (Mizuta and Ventura 1976; Fook et al. 2005; Sivakumar et al. 2005). On the contrary trypsin inhibitor purified from *A. kalkora* seeds showed the presence of two disulfide linked polypeptide chains and a high molecular mass (Zhou et al. 2008). The dissociation constant (K_i) as revealed by Dixon's plot for the inhibitor from *A. lebbeck* was found to be low. A low K_i value indicated high potency of the inhibitor towards the enzyme. Earlier trypsin inhibitors with low value of K_i were reported from *Poecilanthe parviflora* (Garcia et al. 2004), *Calliandra selloi* Macbride (Yoshizaki et al. 2007), *Albizia kalkora* (Zhou et al. 2008) and *Cassia obtusifolia* (Liao et al. 2007). The analysis of Lineweaver-Burk plot showed the inhibitor to be of non-competitive type like trypsin inhibitors from *Adenanthera pavonina* (Macedo et al. 2004), *Vicia faba* (Gupta et al. 2000), and *Tamarindus indica* (Araujo et al. 2005). Shee and Sharma (2007), Oliveira et al. (2007) and Bhattacharyya et al. (2006), however found the inhibitors to be of competitive type. The inhibitory activity of the purified trypsin inhibitor was retained over a wide range of temperature (20–60°C). Trypsin inhibitors purified from seeds of *Caesalpinia bonduc* and *Archidendron ellipticum* were also found to be heat tolerant up to 60°C (Bhattacharyya et al. 2007; Bhattacharyya et al. 2006). Two pH optima at 7.5 and 9.0 were obtained for the purified trypsin inhibitor during the present studies. Maggo et al. (1999), also reported two pH optima for the purified trypsin inhibitor from rice bean (pH 6 and 10). The purified trypsin inhibitor was found to be susceptible to varying concentrations of reducing agents viz., 2-mercaptoethanol and Dithiothreitol. This could be explained by the fact that the three dimensional structure of the inhibitor is stabilized by disulfide bonds which get reduced in the presence of reducing agents. Significant reduction in trypsin inhibitor activity after incubation with 2-mercaptoethanol was also reported in *Phaseolus mungo* (Hajela et al. 1999), *Vigna umbellata* (Maggo et al. 1999) and *Vicia faba* (Gupta et al. 2000). Trypsin inhibitors from *Calliandra selloi* Macbride (Yoshizaki et al. 2007), *Lens culinaris* (Cheung et al. 2007) and *Dolichos biflorus* (Ramasarma et al. 1995) were also found to be susceptible to DTT treatment. However, trypsin inhibitors purified from *Adenanthera pavonina* L. and *Poecilanthe parviflora* were found to be stable in the presence of DTT (Macedo et al. 2004; Garcia et al. 2004). Susceptibility to reducing agents, thermostability and lower molecular

weight of the purified inhibitor indicated it to be classified as Bowman-Birk inhibitor. However, sequencing of *Albizia lebbeck* Bowman Birk Inhibitor would ultimately confirm the inhibitor to belong to BBI family of inhibitors. Similarly, other Bowman-Birk inhibitors showing low molecular weight, thermostability and susceptibility to reducing agents have been isolated, purified and characterized from *Vigna mungo* (Prasad et al. 2010), *Lupinus albus* (Scarafoni et al. 2008) and *Apis americana* (Zhang et al. 2008). Several protease inhibitors have been reported to exhibit inhibitory activity against insect proteases (Reed et al. 1999). Toxicity of the purified inhibitor was checked by its inhibitory activity towards digestive gut proteinases extracted from Lepidopteran insect *Spodoptera littoralis*. Inhibition of gut protease of *Spodoptera frugiperda* was reported by trypsin inhibitor from *Poecilanthe parviflora* (Garcia et al. 2004), *Crotalaria pallida* (Gomes et al. 2005) and *Tamarindus indica* (Araujo et al. 2005). *In vivo* feeding tests were carried out to investigate the potential role of purified trypsin inhibitor as a defence factor against *Pieris brassicae* and observed suppressed larval growth followed by mortality. This may be due to direct inhibition of digestive enzyme and depletion of essential amino acids for larvae. Inhibition of gut trypsin of *Pieris rapae* larvae by purified trypsin inhibitor was reported from *Cassia obtusifolia* (Liao et al. 2007) and *Albizia kalkora* (Zhou et al. 2008).

Conclusion

Considering the high complexity of protease inhibitor interaction in host pest system and the diversity of proteolytic enzymes used by pests and pathogens to hydrolyze dietary proteins or to cleave peptide bonds in more specific processes (Graham et al. 1997), the choice of an appropriate protein inhibitor or set of protein inhibitors represents a primary determinant in success or failure of any pest control strategy relying on protease inhibition. The availability of diverse genes from different plant species makes it a possibility to use one or more genes in combination, whose products are targeted at different biochemical and physiological processes within the insect. These packages will not only contain protease inhibitor genes but also lectins, α -amylase inhibitors or other plant genes encoding insecticidal proteins. This technology may not replace the use of chemical pesticides in near future but effectively complement it.

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